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Original Article

HPLC profile and simultaneous quantitative analysis of tingenone and pristimerin in four *Celastraceae* species using HPLC-UV-DAD-MS

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ABSTRACT

A validated method for the identification and authentication of tingenone and pristimerin was developed using HPLC. The chromatographic profile analysis was combined with simultaneous quantification in *Crossopetalum rhacoma* Crantz, *Cassine xylocarpa* Vent, *Semialarium mexicanum* (Miers) Mennega (known as cancerina), and *Maytenus phyllanthoides* Benth, through microwave-assisted extraction. The HPLC profiles of the four analyzed species showed three similar signals, which corresponded to the main chemotaxonomic markers of the Celastraceae family: quinonemethide triterpenes. HPLC profile analysis was used as a tool to identify the relationship with the quinonemethide triterpenes, for establishing the taxonomic position of some species whose placement in, or within, the Celastraceae family is uncertain.

Introduction

The plant family Celastraceae includes more than 1200 species distributed globally (Simmons et al., 2012), with most members found in tropical and subtropical regions. Plants belonging to the Celastraceae family are utilized for different purposes in traditional medicine; for acetylcholinesterase inhibition (Ferreira et al., 2017), and as analgesic and anti-inflammatory substances. In addition, they are not genotoxic, and facilitate antigenic (De Oliveira Meneguetti et al., 2015), antimitotic (Morita et al., 2008), antiulcerogenic (De Andrade et al., 2007), antibacterial (Alvarenga et al., 1999), antiparasitic (Figueiredo et al., 1998; Mena-Rejón et al., 2007), and antiviral (Osorio et al., 2012) activities. Their use as pesticides is also common (Avilla et al., 2000). A pharmacokinetic study (Zhang et al., 2012) reported the synthesis of quinonemethide triterpene (QMT) analogues (Klaic et al., 2012), and highlighted the antinociceptive peripheral mechanism of tingenone (**1**) action, from opiodergic activation, and nitric oxide pathways (Veloso et al., 2017). QMT are responsible for such pharmacological activities, and have been identified as chemical markers of the Celastraceae family (González et al., 2000).

Certain Celastraceae taxa (previously classified as other genera) have been removed from the family. Currently, taxa that do not exist or have been incorporated into a similar or new genus include the genera *Gymnosporia* and *Rhacoma*, which have been included into *Maytenus* (Qin et al., 2008) and *Crossopetalum* respectively. *Crossopetalum rhacoma* incorporates two different genera and is included in the same species and family Celastraceae. The taxonomic position of the Hippocrateceae is also under discussion to determine whether it should be included in the Celastraceae family or not (Simmons and Hedin, 1999). The botanical features of the two families suggest that there are unique morphologies distinguishing them from one another, however, chemical studies have determined that both belong to the same family (Celastraceae), considering the isolation of the metabolites, including QMT, dulcitol, and 1,4-transpolyisoprene (Mena-Rejón et al., 2007).

In a previous study, Araujo-León and colleagues revealed the first fingerprint analyses of QMT in barks of Semialarium mexicanun (wild vs commercial roots bark) utilized in traditional Mexican medicine. The methodology developed in the previous work can be utilized for the quality control of the discussed herbal root bark. In Mexico, however, there are several other genera that were not included in the fingerprint analyses. This study aimed to carry out a comparison of five different Celastraceae species from Yucatán, Mexico.

Microwave Assisted Extraction (MAE), gave the best extraction yield, and was used instead of the traditional extraction techniques (Boutaoui et al., 2018a,b; Mocan et al., 2018), which included the Soxhlet technique, sonication, heating under reflux, and maceration (Ong, 2004; Mollica et al., 2017). Since these techniques require

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large quantities of organic solvents and long extraction times, they exhibit poor extraction efficiencies, and in addition, several environmental and human health issues associated with their use have been reported recently (Heng et al., 2013).

Our work was performed with the proposition that phylogenetic relationships within the Celastraceae family can resolve questionable affinities among taxa (Simmons et al., 2012). Thus, the main objective was to identify and quantify the QMT in five members of the Celastraceae family, using significantly smaller amounts of plant material (0.5 g), and to help establish the taxonomic position of other species whose inclusion in the Celastraceae family is questionable. Fingerprint analysis, and the presence/absence of chemotaxonomic markers (QMT) were used to complete our research.

Materials and methods

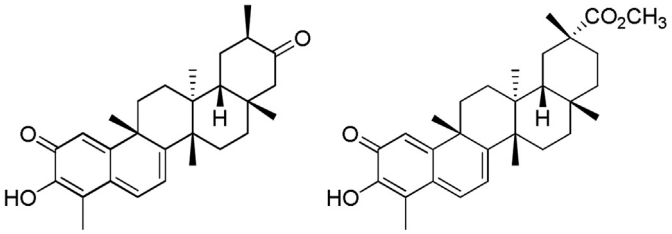
Chemicals and reagents

The extraction solvent, hexane, was distilled in our laboratory and identified by Gas chromatography–mass spectrometry (GC–MS). The methanol (J. T. Baker, USA) and tetrahydrofuran (EM Science, USA) used in the chromatographic analysis had chromatography grade. Deionized water was purified using an E-pure water purification system (Thermo Scientific, USA). The phosphoric acid (high purity; Monterrey, N.L., México) and acetic acid (>99%; Sigma-Aldrich, USA) used in the study were of analytical grade (ACS).

In previous work (Araujo-Leon et al., 2015), it was determined that the best extraction yield, with high selectivity for QMT compounds, was obtained using hexane. Thus, hexane was used as the extraction solvent in the work reported here.

Microwave assisted extraction

A CEM MARS 5 Microwave Accelerated Reaction System (CEM Corporation, Matthews, NC, USA) was employed for extraction. A 0.5 g portion of target root was loaded into each extraction cylinder, with 50 ml of solvent. The microwave power and duration were set to 90 W, and 15 min, respectively. Temperature was uncontrolled. The refinement step, in which some fatty acids and molecules with high affinity for C18 were eliminated, was carried out in accordance with the report published by Araujo-Leon and colleagues (Araujo-Leon et al., 2015). The extracts were then resuspended in MeOH (2 ml) for High-Performance Liquid Chromatography (HPLC) with UV Diode-Array Detection Mass Spectrometry (HPLC-UV-DAD-MS) analysis. Tingenone **(1)** and pristimerin **(2)** were quantified using an external standard calibration curve.



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Validation

The method was validated following the International Conference on Harmonization (ICH) Q2A guidelines with respect to specificity, accuracy, precision, linearity range, limit of detection (LOD), limit of quantification (LOQ), and robustness.

Linearity range

The linearity was studied using six different amounts of tingenone **(1)** and pristimerin **(2)** (within the range 10–1000 μg/ml). A calibration curve was also generated using a linear regression of a plot of the peak area against the amount injected into the HPLC column.

LOD and LOQ

The LOD and LOQ could be calculated to create a new calibration curve (8–12 μg/ml), using the parameters shown in equation (1)

$$L = \frac{k\left(S_{y,x}\right)}{b\sqrt{n}} \quad (1)$$

In equation (1), L is the LOD or LOQ, k is a constant *(i.e.* the value is 3 for the LOD, and 10 for the LOQ), S_{yx} is the residual standard deviation (SD), and b is the gradient.

Precision and recovery

Recovery was calculated using the percentage of bias. The peak areas of the tingenone and pristimerin standards were compared with the matrix recovery. The precision was evaluated using the relative standard deviation (RSD) of the pristimerin signal in the matrix. Evaluations were performed intraday ($n = 9$), and interday ($n = 27$). The root bark was spiked with a pristimerin solution at 10, 250, and 1000 μg, in 70%, 100%, and 130% root bark solutions, respectively.

HPLC-UV-DAD instrumentation

An HPLC system (Agilent Technologies 1200-series, Agilent, San Jose, CA, USA), with a quaternary pump and a UV-DAD detector equipped with a C18 column (250 mm × 4.6 mm, internal diameter 5 μm, Zorbax Eclipse Plus, Agilent, USA), was used. Chromatography was performed under gradient conditions (Araujo-Leon et al., 2015), with H_2O:MeOH:THF. The water contained 1% H_3PO_4, and the flow rate of the mobile phase was 1.5 ml/min. 5 μl of the sample was injected. The column was purged with the mobile phase for 10 min, followed by equilibration for 10 min, and then 40 min were required for sample analysis. Spectral data were collected at detection wavelengths of 254 nm and 420 nm, and the data collected at 420 nm were plotted.

HPLC-MS instrumentation

A binary pump (Agilent Technologies 1200-series, Agilent, San Jose, CA, USA) coupled to a mass spectrometer (Esquire 6000 IT, Bruker Daltonic GmbH, Bremen, Germany) equipped with an ESI source (operated in positive mode), was utilized. The IT mass spectrometry parameters were set as follows: Nebulizer to 40 psi; drying gas flow to 10 l/min; temperature to 300 °C; and capillary voltage to 4000 V. Spectra were recorded in positive-ion mode, between m/z 100 and 1000. The LC–MS system was equipped with a C18 column (100 mm × 2.1 mm, internal diameter 3.5 μm; Zorbax Eclipse Plus, Agilent, USA). Chromatography was performed under gradient conditions with MeOH:H_2O (Araujo-Leon et al., 2015). The water contained 0.1% acetic acid, and the mobile phase flow rate was 0.2 ml/min. The column was purged with MeOH, followed by equilibration for 10 min with the initial mobile phase. Sample analysis took 40 min.

Plant material

A voucher specimen for each plant *Maytenus phyllanthoides* Benth. (N 21° 17.9′, W 89° 33.5′); *Crossopetalum rhacoma* Crantz (N 21° 17.9′, W 89° 33.5′); *Semialarium mexicanum* (Miers) Mennega

Table 1
Calibration parameters for tingenone (**1**) and pristimerin (**2**).

Parameters	Tingenone	Pristimerin
$a + t_{(n-2)}s_a$	4.51 ± 0.3664	4.04 ± 0.5487
$a + t_{(n-2)}s_a$	1.36 ± 17.1318	−4.17 ± 5.5125
$S_{y/x}$	1.08	1.38
r	0.9917	0.9925
Lack of fit	0.9060	0.9119
LOD (μg/ml)	0.42 (2.1 pg)	0.59 (3.0 pg)
LOQ (μg/ml)	1.38 (6.9 pg)	1.98 (9.9 pg)

Table 2
Tingenone (**1**) and pristimerin (**2**) concentrations in five species of the Celastraceae family.

Species	Percent in crude extract		Root bark (mg)	
	Tingenone	Pristimerin	Tingenone	Pristimerin
Cancerina (*S. mexicanum*)	1.43%	5.77%	0.07 ± 0.01	1.23 ± 0.05
C. rhacoma	6.07%	71.94%	0.81 ± 0.09	9.64 ± 0.12
C. xylocarpa	5.75%	3.33%	0.31 ± 0.07	0.18 ± 0.03
S. mexicanum	12.65%	27.95%	0.56 ± 0.03	0.31 ± 0.06
M. phyllanthoides	6.69%	4.43%	0.47 ± 0.10	0.30 ± 0.08

(N 20° 51.8′, W 89° 38.4′); *Cassine xylocarpa* Vent. (N 20° 51.03′, W 90° 11.48′); and Cancerina (commercial sample of *S. mexicanum* from "Sonara's market – Mexico City") (N 19° 25.1′, W 99° 28.8′) was deposited in the "Alfredo Barrera Marin" Herbarium, of the Universidad Autónoma de Yucatán.

Results and discussion

Validation

Linearity, LOD, and LOQ

For statistical analyses, the slope and intercept were calculated using Student's t-test, with a confidence interval, residual variance, and lack-of-fit test applied, using ANOVA (Table 1). The results pertaining to the linearity were satisfactory, based on graphical examination. A proportional increase was exhibited as the concentration increased, with a correlation coefficient of >0.99. Student's *t*-tests ($t = 2.16$) for the slope were also performed, and the experimental *t* value showed that the slope was not zero. The intercept showed a lower value compared to the reference, whereas the coefficient interval crossed the origin. For the latter, the lack-of-fit test ($p > 0.05$) showed significant differences. Based on these results, the model was deemed to be adequate for the analysis. All of the tests confirmed that the linearity of the method was acceptable, in accordance with international guidelines (ICH Q2A).

In the literature, there are few reports on the linearity, LOD, or LOQ of QMT. Roca-Mezquita and colleagues (2016) reported on an isocratic method, with a low slope (0.1123), and LOD and LOQ, for pristimerin, of 0.4079 and 1.2362 μg/ml, respectively. This reported slope was lower than the results obtained in the present work, and the initial LOD and LOQ values were similar. However, a 20 μl loop was used in the reported study, and so the actual LOD and LOQ values were 8.15 and 24.72 ng for pristimerin, which were higher than those obtained in the present work. In 1998, a similar case was reported by Corsino et al. (1998), with LOD and LOQ values of 0.32 and 72.00 μg/ml, respectively, for QMT. These values corresponded to 6.4 ng and 1.4 μg with a 20 μl loop respectively. In addition, Buffa Filho and colleagues (Buffa Filho et al., 2002) reported an LOD for pristimerin of 22.5 ng.

Accuracy and precision

The precision and accuracy of the instrumental and extraction methods were evaluated. Three factors, including accuracy, repeatability (intraday; $n = 9$), and intermediate precision (interday; $n = 27$), were also assessed, for each method. Accuracy was estimated via recovery experiments, by adding the same levels of pristimerin to the root bark. The repeatability and intermediate precision of the instrumental and extraction methods were satisfactory for pristimerin, as the RSD was <10%. Precision was expressed as the RSD, and was calculated using the area of the analyte peaks.

QMT quantification in Celastraceae

QMT quantification was performed, using the MAE system to determine the concentration of each species. A calibration curve was used to determine the QMT concentration (Table 1) for each species. The results showed that *C. rhacoma* had the highest QMT concentration (Table 2). An ANOVA was conducted to conclude whether significant differences ($p < 0.05$) existed among the obtained concentrations, and it confirmed that there were substantial differences ($p < 0.05$) among half of the evaluated concentrations. Therefore, a DMS multiple comparison test was performed, which showed that the cancerina, *C. xylocarpa*, and *M. phyllanthoides* did not exhibit noteworthy differences ($p > 0.05$). However, *C. rhacoma and S. mexicanum* showed significant differences ($p < 0.05$), with respect to the other species.

QMT quantification in the five Celastraceae species has not been reported in the literature. The genus that has been studied most in this family is *Maytenus*, due to its antioxidant capacity (De Freitas Formenton Macedo Dos Santos et al., 2010), and its tingenone **(1)** and pristimerin **(2)** contents were reported to be 3.84% and 14.0% respectively. To evaluate cytotoxicity activity (Nossack et al., 2004), the leaves and root bark were compared, and the hydro-alcoholic extract of the root bark contained 1.24 mg/g and 0.44 mg/g of tingenone and pristimerin respectively. It was revealed that the root bark is a potential source of antitumor compounds (Buffa Filho et al., 2004). In terms of the QMT derivatives content in cell-tissue cultures and in natural plants, the percentage of QMT has been reported to be higher in a callus than in natural root bark. Finally, five morphological types of *Maytenus ilicifolia* were compared, to quantify the QMT, in an effort to advance biosynthetic studies (Buffa Filho et al., 2002). The results from this study showed that the percentage of pristimerin was comparatively lower than tingenone. This behaviour has been observed in a majority of the research mentioned previously, and also in the present work, with respect to *M. phyllanthoides*.

To our knowledge, Roca-Mézquita et al. (2016) is the only report on the antiprotozoal activity of the dichloromethanolic extract of *C. xylocarpa* (publish as *Elaeodendron trichotomum*) root bark; this extract was composed of 3.84% tingenone **(1)** and 0.14% pristimerin **(2)**.

Evaluation of HPLC profiles in five root barks from the Celastraceae family

Only a few studies concerning the fingerprint chromatograms of Celastraceae members have been conducted. Buffa Filho and colleagues (Buffa Filho et al., 2002) reported five chromatograms from *Maytenus ilicifolia*. In those chromatograms, the two main signals were from maytenin (tingenone), and pristimerin, and were observed in all the chromatograms. In previous work (Araujo-Leon et al., 2015), we reported cancerina (commercial *S. mexicanum* from

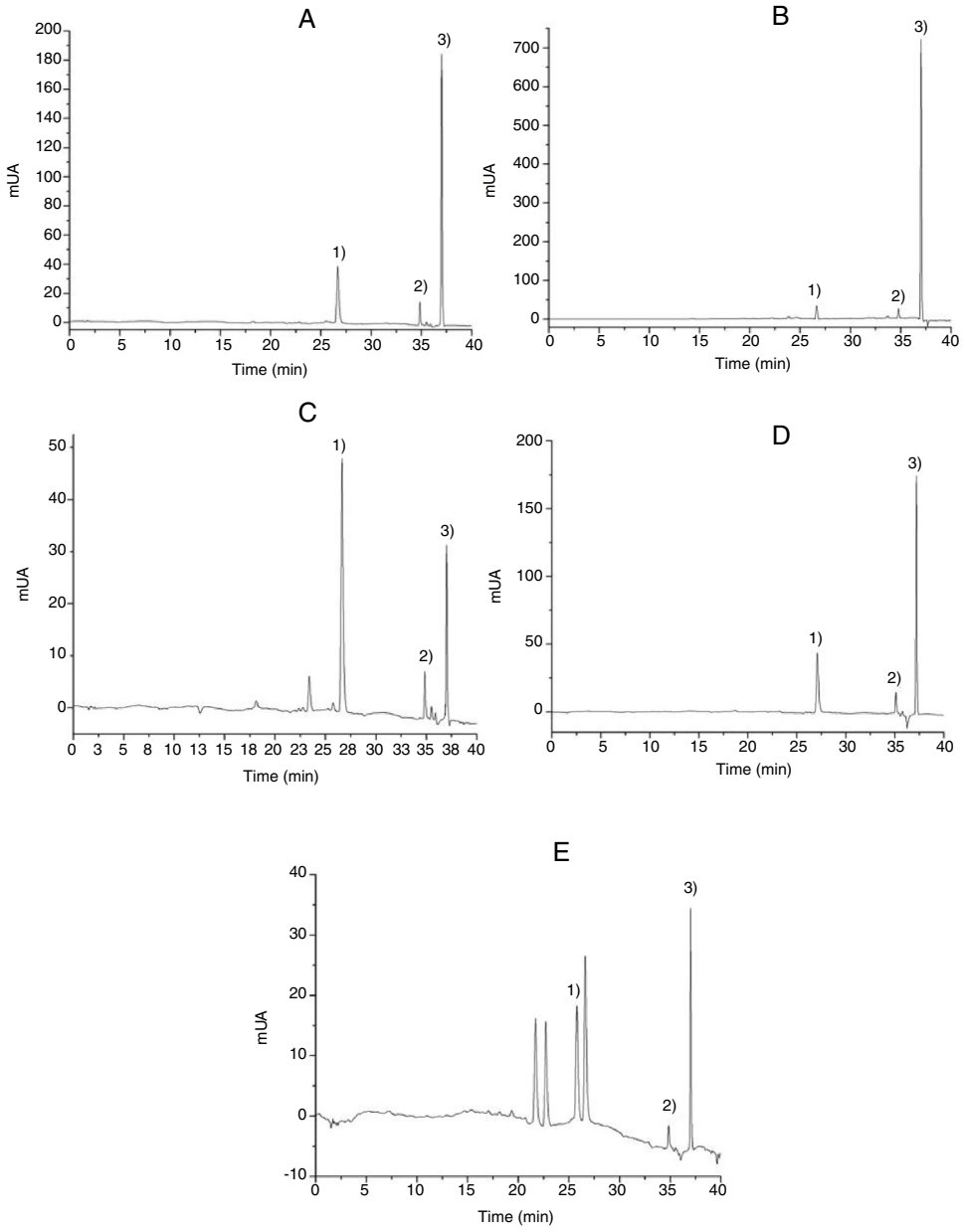

Fig. 1. Chromatograms of (A) Cancerina (commercial sample of *Semialarium mexicanum*); (B) *Crossopetalum rhacoma*; (C) *Cassine xylocarpa*; (D) *S. mexicanum*; and (E) *Maytenus phyllanthoides* root bark, at a detection wavelength of 420 nm.

"Sonara's market – Mexico City") and *S. mexicanum* fingerprints that exhibited similar signals.

The HPLC profile analyses of the five *Celastraceae* species were collected at 420 nm, with the HPLC-UV-DAD system. This particular wavelength was selective for QMT, due to the conjugation in rings A and B in the QMT structure (Araujo-Leon et al., 2015; Buffa Filho et al., 2002). In addition, the identification signal by electrospray ionization/mass spectrometry (ESI/MS), in positive-ion mode, was confirmed, by monitoring the total ions over the m/z range between 100 and 1000.

The HPLC profile (Fig. 1) showed the same three signals reported in the literature. These signals were identified as (1) tingenone, (2) netzahualcoyene, and (3) pristimerin, with retention times of 27.3 ± 0.2 min, 35.3 ± 0.1 min, and 37.6 ± 0.2 min, respectively. The signals were present in the UV spectrum. A maximal absorbance between 410 nm and 440 nm was also observed – a major characteristic of QMT. In the mass spectra, the base peak of each signal corresponded to the molecular weight of the sodium ion adduct (M+23). Furthermore, an ion at $m/z = 201$ was observed in all mass spectra – another characteristic of QMT fragmentation into a tropylium ion (Rodrigues-Filho et al., 2002; Paz et al., 2013).

The results of the fingerprint analyses (Table 3) showed that the relative peak areas of tingenone, in *M. phyllanthoides* and *C. xylocarpa*, were 151% and 172% respectively, which is higher than those of the pristimerin signal. Such discrepancies can aid in differentiating the remaining species, based on high relative areas of pristimerin, as opposed to those concerned with other signals. To the best of our knowledge, this is the first report on the above-mentioned values.

Table 3
Results of the fingerprint analyses ($n = 5$).

Peak number	Relative retention time					Relative peak area				
	1	2	3	Average	RSD (%)	1	2	3	Average	RSD (%)
Cancerina (S. mexicanum)										
1	0.731	0.732	0.728	0.730	0.30	24.99%	23.56%	25.86%	25%	4.68
2	0.947	0.951	0.945	0.948	0.27	9.10%	10.12%	11.04%	10%	9.61
3	1.004	1.004	1.009	1.005	0.28	100.00%	100.00%	100.00%	100.00%	0.00
Crossopetalum rhacoma										
1	0.719	0.727	0.723	0.723	0.52	8.33%	8.04%	8.93%	8%	5.38
2	0.960	0.971	0.949	0.960	1.15	5.88%	5.29%	6.04%	6%	6.89
3	0.999	1.001	0.999	1.000	0.10	100.00%	100.00%	100.00%	100.00%	0.00
Cassine xylocarpa										
1	0.746	0.743	0.734	0.741	0.83	173.09%	171.45%	172.65%	172%	0.49
2	0.945	0.946	0.945	0.945	0.08	32.36%	30.64%	31.96%	32%	2.85
3	0.999	1.001	1.002	1.001	0.13	100.00%	100.00%	100.00%	100.00%	0.00
Semialarium mexicanum										
1	0.720	0.720	0.707	0.716	1.09	44.49%	45.28%	45.98%	45%	1.65
2	0.941	0.939	0.940	0.940	0.06	10.20%	11.29%	11.86%	11%	7.60
3	0.999	1.001	1.002	1.001	0.13	100.00%	100.00%	100.00%	100.00%	0.00
Maytenus phyllantoides										
1	0.745	0.744	0.707	0.732	3.02	146.55%	156.44%	150.15%	151%	3.31
2	0.940	0.935	0.940	0.938	0.32	12.23%	14.32%	13.11%	13%	7.94
3	0.999	1.000	1.002	1.000	0.15	100.00%	100.00%	100.00%	100.00%	0.00
Pristimerin (STD)	1.000	1.000	1.000	1.000	0.00	N/A	N/A	N/A	N/A	N/A

N/A, not apply.

Conclusion

This study is the first to successfully establish an HPLC profile, as well as simultaneous quantification of tingenone **(1)** and pristimerin **(2)**, and chromatographic profiles of QMT, for five species of the Celastraceae family, using root bark samples. The results reveal that the main chemotaxonomic markers in these species are tingenone, netzahualcoyene, and pristimerin. The method developed for this study demonstrates a decent linearity, excellent reliability (sensitivity), and high accuracy and precision. When combined with the fingerprint, this method (using QMT as chemotaxonomic markers) has potential to be used as a tool for the discrimination and authentication of some species whose taxonomic place in the Celastraceae family is currently questionable.

Author's contribution

JAA-L and ZOC-C conceived and designed experiments. JAA-L made all the experiments as part of Master thesis. DVR-C and TIC-M developed the HPLC-DAD and HPLC-MS method and conducted measurements. JAA-L wrote the manuscript. All authors read, commented, and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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